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Characterization of Various Classes of Protein Adducts

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Analysis of the types of protein adducts formed by chemical carcinogens indicate that adducts may be categorized into various classes according to the nature of the carcinogen as well as the amino acid with which they react. Tryptophan(214) of serum albumin was previously shown to react specifically with *N*-sulfonyloxy-*N*-acetyl-4-aminobiphenyl. The same residue is now shown to also react with the sulfate esters of *N*-hydroxy-*N*-acetyl-2-aminofluorene and *N*-hydroxy-*N*,*N*′-diacetylbenzidine. Thus, Trp-214 appears to be a binding site for a variety of activated *N*-aryl hydroxamic acids. Epoxides and diol epoxides derived from polynuclear aromatic hydrocarbons alkylate carboxylic groups in hemoglobin and serum albumin. Because the esters formed are readily hydrolyzed to dihydrodiols and tetrahydrotetrols which can be determined by GC–MS, it is possible to analyze for a wide range of polyaromatic hydrocarbon (PAH) epoxide adducts. With this approach it was shown that human subjects experiencing exposure to ambient levels of environmental PAH do take up and metabolize chrysene and benzo[*a*]pyrene. Feral, bottom-dwelling fish inhabiting contaminated waters were also examined. Globin adducts containing certain dihydroxy groups such as those arising in *anti*-diol epoxide adducts were concentrated by boronate affinity chromatography and further analyzed by HPLC with diode-array UV/visible detection. Four compounds were detected that exhibited spectra characteristic of a polynuclear chromophore. Two of these appeared to be isomers. Further instrumental analysis is needed to elucidate the structure of these unknown putative adducts. A discussion of how these analyses might be conducted as well as their extension to less heavily adducted human globin samples is presented.

Introduction

Carcinogens or their electrophilic metabolites interact with proteins [recently reviewed in Skipper and Tannenbaum (*1*)] as well as DNA. It has been shown that the amount of a protein–carcinogen adduct formed may be used as a quantitative measure of exposure to the carcinogen. The precision of this method will vary for different carcinogens depending on the extent of interindividual differences in uptake and disposition. Similarly, protein adduct levels have also been shown to reflect genetic damage, and it may be assumed that the degree of correlation between adducts and genetic damage will also depend on the extent to which interindividual differences contribute to the disposition of the compound. Because they are more readily obtainable than DNA adducts, protein adducts have an important role in both exposure assessment and genotoxic effects assessment.

Two proteins, hemoglobin and serum albumin, have so far been the nearly exclusive focus of research efforts both in chemistry and epidemiology. Hemoglobin was the first protein proposed for the purpose of molecular dosimetry (*2*), and numerous subsequent studies have borne out its utility. Human exposures to ethylene oxide (*3*), aromatic amines (*4*), benzo[a]pyrene (BaP) (*5*), and the tobacco-specific nitrosamines NNN and NNK (*6*) can now be determined routinely. It has even been possible in the case of 4-aminobiphenyl to demonstrate the influence on adduct levels of a metabolic phenotype known to be a risk factor (*7*). Serum albumin has also proven to be a good target protein for dosimetry in human populations, particularly for aflatoxin B_1 (*8,9*). Serum albumin adducts may also prove to be useful for the dosimetry of the food pyrolysis heterocyclic amines because these compounds may have the same sulfinamide structure as the hemoglobin–aromatic amine adducts (*10*).

The analytical methods developed for these studies tend to be highly specific for a compound or a narrow class of compounds. Ethylene and propylene oxides form *N*-hydroxyethylvaline and *N*-hydroxypropylvaline, respectively, which are selectively cleaved by a modified Edman degradation (*11*). Aromatic amine sulfinamide adducts are cleaved by base-catalyzed hydrolysis, and the product amines are separated by solvent extraction. NNN and NNK adducts also undergo base-catalyzed hydrolysis, releasing 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), but the subsequent isolation procedure is quite different (*6*). Benzo[*a*]pyrene diol epoxide adducts are cleaved by spontaneous hydrolysis facilitated by enzymatic proteolysis, and the product

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tetrahydrotetrols are isolated by monoclonal antibody immunoaffinity chromatography (*12*). Aflatoxin adducts are isolated as a modified lysine residue from thoroughly proteolyzed albumin by immunoaffinity chromatography (*8,9*).

In this paper we explore the possibility that certain procedures developed for specific adducts can be generalized to permit the detection of additional similar but undetermined adducts. Recent results obtained in our laboratories are described, and we attempt to elucidate some of the forces governing the interaction of carcinogens with proteins in an effort to indicate directions for future research.

Protein Binding Sites

The binding of a ligand by its protein receptor is commonly thought to be a highly specific interaction, as is antibody–antigen binding. In such cases, it is often possible to describe the binding site in quite specific terms with respect to the functional groups that interact with the ligand and their spatial orientation, as well as other dimensional features. As applied to the interaction of proteins with carcinogens, the concept of binding sites has been drawn more loosely. Three related but distinct definitions may be made, which will form the basis for the discussion to follow.

First, because the formation of a carcinogen–protein adduct involves the formation of a covalent bond to an amino acid residue in the protein, it may be useful to think of the binding site as the specific residue that undergoes reaction. A second approach is to define a binding site as any one of the 10 different functional groups (hydroxyl, amine, thiol, thioether, carboxyl, carboxamide, guanidine, imidazole, phenol, or indole) found on amino acid side chains, irrespective of its location in the polypeptide chain. Third, a binding site may be described as a region of the protein without reference to any specific residue. As we will show, each of these definitions has different ramifications for the development of methods to detect and identify new carcinogen adducts.

The Binding Site As a Specific Amino Acid Residue

Specific Cases. Several examples of carcinogen binding sites defined as a specific residue are now known. The NH_2-terminal valine of hemoglobin is perhaps the best example. The amino group of this valine has been shown to react with a variety of alkylating agents producing *inter alia* *N*-methyl, *N*-ethyl, *N*-hydroxyethyl, *N*-hydroxypropyl, and *N*-hydroxyphenylethyl valines (*11*). Moreover, aldehydes will condense with the NH_2-terminal valine amino group. A prototypical example is the reaction of glucose with hemoglobin, and, although glucose is not a carcinogen, other aldehydes with potential genotoxic activity such as malondialdehyde also will react with hemoglobin in the same way (*12*).

It has been particularly useful to conceive of the NH_2-terminal amino group as a binding site. By so doing, a generalized method for adduct separation and purification became apparent since the peptide bond in polypeptides can be sequentially cleaved through the Edman procedure. Further adduct concentration was then accomplished by modification of the Edman procedure, which made it more selective for the alkylated form of valine, thereby reducing the amount of nonalkylated valine accompanying the adducts.

Another specific amino acid that has been shown to participate in reaction with a number of carcinogens is the cysteine(93β) of hemoglobin. Fourteen aromatic amines have been identified as having reacted with human hemoglobin *in vivo* (*4*), and, although the exact structure has only been determined in one case (*14*) and deduced from circumstantial evidence in another (*15*), it is almost certain, considering the chemistry of the aromatic amine-hemoglobin interaction, that the other 12 adducts have the same structure. All the amine adducts detected thus far have been formed by monocyclic or bicyclic amines. Evidence is lacking for the presence of larger aromatic amine adducts in human hemoglobin.

The known aromatic amine adducts at the Cys-93β can all be determined by a common procedure. It is probably safe to generalize and say that many other aromatic amine exposures could be detected by application of the same procedure. Once again, the reason that a particular procedure can be extended to the analysis of other adducts is that the procedure used cleaves a bond linking the adduct, a small molecule, to the protein, which is a macromolecule. This principle finds application repeatedly; other examples will be described below.

The single tryptophan residue of serum albumin is also a binding site for a specific class of carcinogens. It was previously reported that this amino acid reacts *in vivo* in the rat with a metabolite of 4-aminobiphenyl, *N*-sulfonyloxy-*N*-acetyl-4-aminobiphenyl (AABP), to form an adduct with a stable tryptophan–aminobiphenyl linkage (*16*). We have now shown that the same adduct is formed by reaction of synthetic sulfate ester of *N*-hydroxy-AABP with human serum albumin *in vitro*. The essential role of sulfation was demonstrated through additional *in vitro* experiments with hepatocyte preparations. Coincubation of rat hepatocytes with human serum albumin and *N*-hydroxy-AABP generated the tryptophan adduct, and the yield was greatly decreased by the omission of sulfate from the medium or by the inclusion of the sulfotransferase inhibitor dichloronitrophenol.

Likewise, it has been shown that the serum albumin tryptophan reacts with the sulfate esters of *N*-hydroxy-*N*-acetyl-2-aminofluorene (AAF) and *N*-hydroxy-*N,N'* diacetylbenzidine (dAB_2) (F. F. Kadlubar, personal communication). Pronase digests of serum albumin reacted with the synthetic sulfate esters produced one major HPLC peak in each case with retention time similar to that of the AABP adduct peak. The same adduct peaks were produced when the hydroxamic acids were incubated with hepatocytes and serum albumin, and their yield could be controlled by the amount of sulfate and sulfotransferase inhibitors in the medium.

Fast-atom bombardment (FAB)–MS was used for structure determination. Pronase digestion yielded a tetrapeptide from AABP-adducted serum albumin with the sequence Ala-Trp-Ala-Val. An intense $(M+H)^+$ ion (m/z 655) was observed in the mass spectrum of this adduct corresponding to the peptidyl residue (444) plus an acetylaminobiphenylyl group (210). Other ions that resulted from various peptide chain fragmentations were also observed, and these ions were helpful in confirming the structure assignment.

When the Pronase fragments corresponding to AAF and dABz adducts were subjected to FAB-MS, $(M+H)^+$ ions were observed at m/z 667 and m/z 712, respectively, which correspond to adducts of Ala-Trp-Ala-Val. As with the AABP adduct, appropriate fragment ions were also observed.

These data suggest that nitrenium ions generated from aromatic hydroxamic acids exhibit a high degree of selectivity for the tryptophan residue in human serum albumin. Modeling of the protein also suggests that there may be significant size and/or shape constraints on the structure of the hydroxamic acids. These two properties may combine to define a quite selective binding site.

Qualitative Analysis of Additional Adducts. Identification of a specific residue as participating in reaction with carcinogens may provide the basis for designing approaches to the identification of other adducts, as illustrated in the following example drawn from research in K. Biemann's laboratories. Cysteine (93β) of hemoglobin reacts with certain electrophilic species known as Michael acceptors to form stable products. Acrylamide (*17*) and 2-vinylpyridine are two good examples. The molecular weight, at least, of other adducts could be determined through the following generic approach.

Cysteine(93β)-adducted hemoglobin can be separated from native hemoglobin chromatographically (*18*). Trypsinolysis leads to the production of a peptide containing the 93β residue (83–95), which can easily be separated from the other tryptic fragments. The adducted tryptic fragments are then introduced into a tandem mass spectrometer for analysis. By monitoring MS-2 for fragment ions characteristic of the tryptic fragment and stepping MS-1 through a range of mass values appropriately greater than the tryptic fragment, it is possible to determine the molecular weights of adducted tryptic fragments.

Clearly, additional information would be needed to fully elucidate structure. Nonetheless, the feasibility of this important first step has been demonstrated. Other spectroscopic techniques for obtaining further structural information are available and under development. One of these, fluorescence line narrowing, is described in more detail below.

The Binding Site As an Amino Acid Functional Group

Identifying the specific amino acid residue that has reacted with a carcinogen, if indeed it is only one, is usually a challenging task, especially if the adduct is formed by an unstable linkage. Moreover, from a practical point of view this identification may be unnecessary. Thus, in many instances it may be worthwhile to think of a protein binding site for carcinogens as just one of the ten different amino acid side chain functional groups without reference to its location or locations in the protein.

Specific Cases. The earliest studies of protein adduct formation in which strong acid hydrolysis was used to degrade the protein took this approach. Alkylating agents were shown to form N^1- and N^3-alkyl histidines, for example, in addition to *S*-alkyl cysteine. These adducts could be identified because they possessed a very stable carcinogen-amino acid linkage that survived the strong conditions of hydrolysis used to isolate them.

More recently, it has become apparent that many carcinogens form less stable bonds to the amino acids with which they react. Nevertheless, the adducts may be stable *in vivo* as the result of secondary interactions such as van der Waals attraction between the carcinogen and other residues in the protein. When adducted protein is degraded by denaturation or proteolysis, these secondary interactions are disrupted and the covalent adduct linkage becomes susceptible to hydrolysis.

BPDE (*r*-7,*t*-8-dihydroxy-*c*-9,*c*-10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene), for example, reacts with human hemoglobin mainly by alkylation of aspartate(47) in the α chain (*19*). The product is stable in native hemoglobin, but only dissociation into dimers is necessary to initiate hydrolysis of the product ester. Proof for an ester adduct as the source of BaP tetrol from proteolysis of BPDE-treated hemoglobin was obtained through isotope incorporation studies (*20*). The same approach was used to demonstrate that BPDE alkylation of human serum albumin also yielded a substantial fraction of ester products (*21*). BPDE appears to be typical of the benzylic epoxide metabolites of the PAH class of carcinogens. Although the identity of the acidic amino acids that are alkylated by the PAH epoxides has not been determined except in the case of BPDE and hemoglobin, it appears that esters are a common product and that they decompose to give PAH alcohols upon proteolysis of adducted proteins.

The PAH-derived esters hydrolyze so readily because the carbon atom that alkylates a carboxyl group is attached to an aromatic ring system. When esters with such a structure are hydrolyzed at neutral pH, the carboxyl group becomes a leaving group. Its departure is greatly facilitated by delocalization of the resultant carbonium ion throughout the aromatic rings. Because even a single 6-membered ring is sufficient to effect this mode of cleavage (e.g., 1-phenylethyl esters), it is generally to be expected that PAH epoxides will form esters that spontaneously hydrolyze.

We have taken advantage of this generality to detect adducts of PAH other than BaP in various types of blood specimens. The challenge has been to devise methods for separating the resultant PAH alcohols from the amino acid and peptide milieu generated by proteolysis. One successful procedure has been monoclonal antibody immunoaffinity chromatography. With this technique it has been possible to demonstrate the presence of adducts of chrysene diol epoxide in human hemoglobin specimens (*22*). Tentative evidence for the presence of several other diol epoxide adducts has also been obtained (*23*). Equally important is the absence of tetrols that are known to cross-react with the antibody used, for their absence may be taken as a meaningful indication that, although the individuals examined were exposed to the parent hydrocarbon as a result of its presence in the environment, the hydrocarbon was not metabolized to a diol epoxide. Tetrols that are conspicuously absent include the 7,8,9,10 tetrols originating from the *syn* isomer of BPDE as well as any tetrols originating from benz[*a*]anthracene diol epoxides.

Another separation scheme that has been used is affinity chromatography with boronate media. This approach is selective for, among others, 1,2-diols, which, if in a ring, are *cis* to each other. Such vicinal diols are present in those tetrols that arise from *anti*-diol epoxides. Boronate affinity chromatography has been applied to digests of globin isolated from feral, bottom-dwelling fish that live in contaminated waters. HPLC analysis of boronate-bound fractions are remarkably uncomplicated, as illustrated in Figure 1.

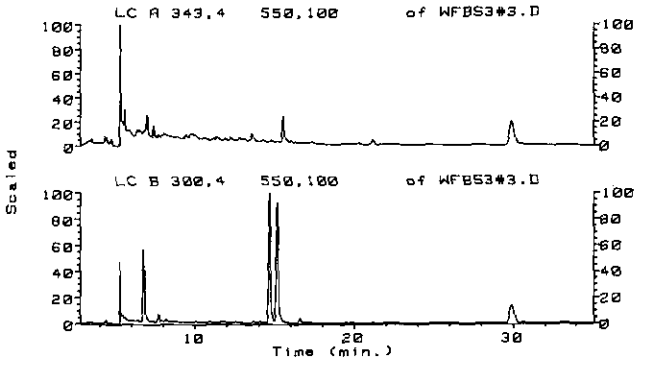

FIGURE 1. HPLC analysis of boronate-bound fraction from winter flounder globin. Sample was prepared be treatment of hemoglobin with acidic acetone followed by Pronase digestion of the precipitated globin. The digest was applied to a boronate affinity column, and the bound material was eluted with pH 3 formate buffer. Upper trace recorded at 343 nm and lower trace at 300 nm.

The chromatogram illustrated in Figure 1 was recorded using a diode array UV/visible detector, and thus spectra were obtained for each peak. Most were uninteresting, but four of them strongly resemble spectra of polynuclear aromatic systems (Fig. 2). Two of the peaks are clearly a pair of isomers exhibiting slight perturbations of the spectrum of what is essentially the same chromophore in each.

To be sure, the chromatographic and spectral evidence is insufficient to identify the four peaks described above as PAH–globin adducts. Nonetheless, these four compounds are sufficiently like what could be expected of such adducts that they warrant further efforts at identification.

Characterization of Adducts in Situ. As indicated above, the process of breaking down an adducted protein into fragments of molecular weight suitable for most qualitative analytical techniques may result in the loss of particularly labile adducts. If the carcinogen possesses a chromophore, though, it may be possible to obtain structural information directly by spectroscopic means. Fluorescence line narrowing (FLN) spectroscopy has been used, for example, to verify the existence BPDE ester adducts in both hemoglobin (*24*) and serum albumin (*25*).

Fluorescence line narrowing produces spectra that are highly distinctive. In the case of BPDE adducts, the spectra are sufficiently characteristic of the substituent present at C-10 that hydroxy and alkoxy, carboxy, alkylamino, imidazolyl, and thioether adducts can all be distinguished from one another. Normal human hemoglobin, when subjected to FLN, exhibited a spectrum characteristic of a C-10 carboxy substituent, consistent with the known presence of ester adducts. In contrast, human serum albumin alkylated with BPDE was more complex, but could be deconvoluted to reveal the spectra of ester and imidazolyl adducts.

The Binding Site As a Region of a Protein

Too little is known about the properties of the binding sites of proteins such as serum albumin or hemoglobin to allow much in the way of generalities to be drawn at present. However, the results obtained from the study of carcinogen binding thus far indicate that considerable caution should be exercised in extrapolating from one case to another. For example, it was recently shown (*21*) that *anti*-BPDE reacts with human serum albumin to form an adduct at His-146. This amino acid is a reactive residue in a previously described binding site for a variety of hydrophobic compounds located in subdomain 1-C (*26*). Rat serum albumin also possesses this histidine residue, yet in a recent study (Tannenbaum et al., unpublished data), we found that it does not react with fluoranthene diol epoxides. Instead, a lysine adduct is produced, formed by reaction with Lys-385, which resides in subdomain 2-C. Because this lysine is replaced by glutamine in human serum albumin, it is not possible to make a definitive conclusion from these two results.

If the subdomain 1-C binding site is the primary binding site, as has been suggested (*26*), then there would appear to be limited substrate specificity because fluoranthene did not react with His-146. Alternatively, our data do not contradict the concept of a more general subdomain 2-C binding site because human

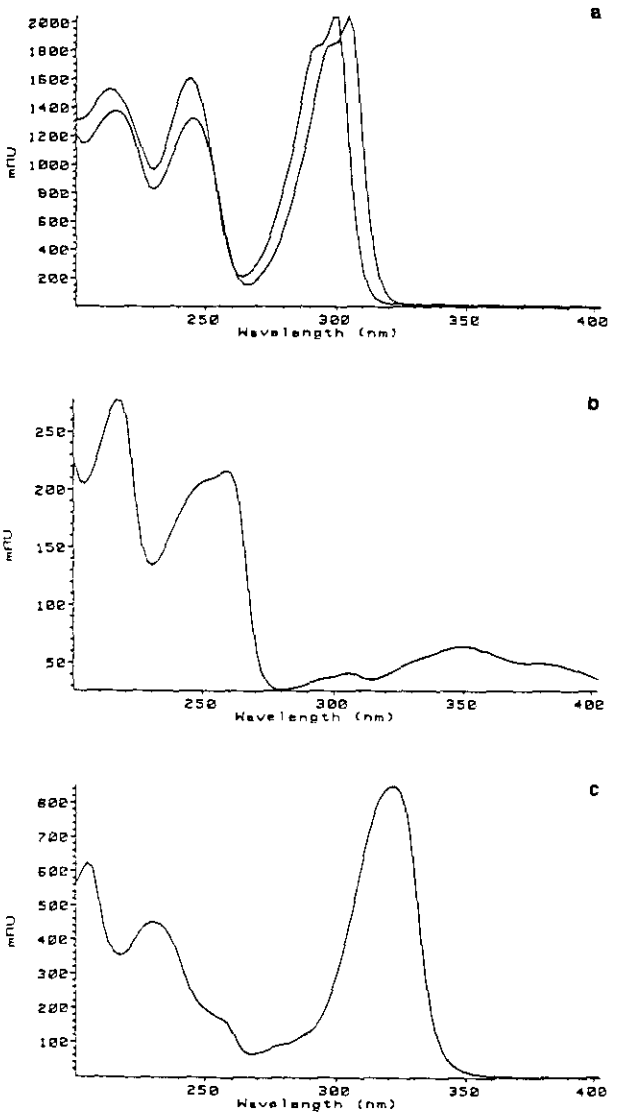

FIGURE 2. UV spectra of the peaks at (*a*) 14.69 and 15.18 min, (*b*) 15.54 min, and (*c*) 29.90 min.

serum albumin does not have a lysine at residue 385, but if this is the case, then once again it is evident that species differences must be clearly recognized when using animal models for human protein binding.

Conclusion

Carcinogen-protein adducts have been shown over the past 15 years to be useful biomarkers in a variety of biomonitoring studies, especially as applied to the study of known or selected chemicals. Further advances in the field may include the analysis of proteins to identify additional generically similar adducts, thereby revealing previously undetermined exposures and verifying or rejecting the participation of certain metabolic pathways in carcinogen metabolism. Among the factors that may make this possible is our increasing understanding of the binding sites of proteins for the electrophilic intermediates produced by the metabolism of procarcinogens.

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